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(54) **Title:** METHOD AND APPARATUS FOR EARLY DIAGNOSIS OF BLADDER TUMOR IN URINE SAMPLES

(57) **Abstract:** A method for early diagnosis of bladder tumor in a urine sample, which comprises a step of amplification of the RNA extracted from cells present in the urine by using a marker for the messenger RNA of the catalytic component of telomerase (hTRT), a marker for β-actin to demonstrate RNA accessibility and as standard for quantitative estimation, in combination with at least one additional marker chosen from the group that comprises: a marker for a protein of the cytokeratin family, a lymphocyte marker which is suitable to detect inflammatory cells associated with neoplastic infiltration, and a final step of detecting the amplified material.

METHOD AND APPARATUS FOR EARLY DIAGNOSIS OF BLADDER TUMOR IN URINE SAMPLES

Technical Field

The present invention relates to a method and an apparatus for early diagnosis of bladder tumor in urine samples.

In particular, the present invention relates to a method and to the corresponding diagnostic apparatus for detecting, from the onset, even initial tumor forms affecting the mucous membrane of the bladder without resorting to the use of more or less invasive methods.

The method according to the invention is particularly suitable for detecting transitional carcinomas of the bladder, which make up almost all of the tumors affecting this region.

Background art

It is known that transitional bladder cell carcinoma (TCC) is one of the most widespread tumor in Western societies. At the time of diagnosis, 20% of TCCs are found to be of the invasive type, while 80% are superficial. 20% of the latter can evolve into an invasive form with infiltration of the muscular tunic of the bladder wall.

Due to the frequent relapses of these forms of tumor, periodic monitoring of patients, including asymptomatic ones, is indispensable.

However, it has been found that cystoscopy, which is currently the most widely used monitoring method because it provides the most reliable results, cannot be considered a method suitable for the screening of a large number of patients due to invasive nature of the procedure, which is particularly unpleasant in male patients.

An alternative non-invasive diagnostic method is cytological examination of urine. This method has the disadvantage of low sensitivity, which allows to recognize the neoplastic lesion only in 40-50% of investigated cases.

Transitional bladder tumor usually affects the elderly population, which

is increasing considerably in developed societies. This leads to the expectation of an increase in the number of requests for diagnosis of these tumors in the future. Since the analyses to which patients are subjected for accurate diagnosis are mostly invasive, an alternative, noninvasive, high-sensitivity and high-specificity method would be very important. Cytology, which is a noninvasive method, has low sensitivity and is also affected by the variability of the interpretation of the people who perform the test. Commercially available alternative methods are based on determining the various tumor-specific antigens (BTA, bladder tumor associated analytes (Bard Diagnostics, Redmond, Washington), NMP 22 nuclear matrix protein [Matritech, Newton, Massachusetts]). Although these methods are more sensitive than cytological examination, their stated clinical usefulness is compromised by the extreme variability due to the inflammatory processes, which can interfere with their sensitivity and specificity.

It is known that molecular techniques, based on DNA and RNA analysis, are extremely sensitive and specific and are therefore suggested increasingly also in clinical diagnostics.

It is well-known that in bladder tumor the pattern of genetic alterations, which cause the development of neoplastic cells, is highly complex, making analysis particularly troublesome. One of the first mutations studied at the level of tumor cells (ras oncogene mutation) has been found in bladder tumor cell lines and rarely in primitive bladder tumor.

Sidransky et al. (Science (1991) 252, 252-253) found point mutations of the oncosuppressor gene p53 in 61% of bladder tumor cases. Due to this relatively low frequency of mutations and to the large number of variations in said mutations, this investigation method cannot be used in clinical diagnostics.

Cytogenetic methods have revealed some chromosomal deletions at 9q, 11p and 17p, where oncosuppressor genes may be located. In order to identify loss of heterozygosity and gene instability, microsatellite markers

have an important application. These genetic alterations at the microsatellite level, however, are occasional, and in a significant number of tumors might not be revealed despite using a large number of microsatellite markers. Mao et al. (Science (1996) 271, 659-662) used a group of 13 tri- and tetranucleotide markers (it has been found that the longest repeats are those subjected to deletions or expansion). 95% of tumor cases (19 out of 20 cases) had an identical clonal alteration in the urine sample and in tumor biopsies.

Although the method has been found to be sensitive, it is not suitable for screening because it is extremely labor-intensive (large number of individuals to be analyzed).

The following diagnostic kits are currently commercially available:

-- BTA (bladder tumor associated analytes), which has 74% sensitivity and 73% specificity. False positives have been found in patients without a bladder tumor but with considerable hematuria (6 out of 7 cases).

-- NMP22 (nuclear matrix protein), which has 53% sensitivity and 60% specificity.

-- FDP (fibrin and fibrinogen degradation products), which has 52% sensitivity and 91% specificity (The Journal of Urology (1999) 161, 388-394).

Currently, the need is felt to have a new method, with better characteristics, for the diagnosis and for following the evolution of bladder tumor, which is highly sensitive and specific, does not require harmful reagents and can be used directly on the urine of patients.

Determination of telomerase activity in cells obtained by bladder washing has recently been proposed (Clin. Canc. Research. (1998) 4, 535-538). Telomeres are the terminal portions of eukaryotic chromosomes and are meant to protect and stabilize chromosomes. Since DNA polymerase is unable to synthesize them during replication of somatic cells, telomeres gradually shorten. When the telomeres reach a limit length, chromosomal

instability leads to cell death (apoptosis). In immortalization of somatic cells and in malignant transformation, the telomeres stabilize and telomerase enzyme activity appears.

Telomerase is the enzyme responsible for telomere synthesis. It is constituted by three components (Current opinion in Genetics and Development (1999) 9, 97-103):

1) the RNA (hTR) component, which acts as a template for telomere synthesis (short repetitive sequences);

2) the catalytic component - hTRT;

3) the telomerase-associated protein (TEP1).

The presence of the two components hTR and hTRT is sufficient to reconstruct telomerase activity in vitro.

Telomerase activity (addition of telomere repeats at the end of chromosomes, which occurs by using as template its RNA component [hTR]) is measured with the TRAP assay (telomeric repeat amplification protocol). Boehringer Mannheim (Roche Biochemicals) has developed a nonradioactive TRAP analysis system based on a method which uses an ELISA-type microplate. Since in excessively concentrated protein lysates the presence of Taq polymerase inhibitors may mask telomerase activity (false negatives), the TRAP assay must be performed by using various dilutions of the same sample. Furthermore, the presence of protease, RNase and the pH variations to which cells are exposed in urine can compromise the efficiency of the assay (false negatives).

Lee et al. (Clin. Can. Res. (1998) 4, 535-538) have demonstrated telomerase activity (TRAP assay) in 96% of tumor cases by performing analysis on tumor tissue and in bladder washes. Specificity is 96%. Heine (Journal of Pathology (1998) 184, 71-76) et al. have shown, with TRAP analysis, telomerase activity in 95% (19 out of 20) of bladder tumor tissues and in 70% (14 out of 20) of bladder washes, while activity was absent in urine sediment of bladder tumor patients. Rahat et al. (Cancer (1999) 85,

919-924) have demonstrated telomerase activity with TRAP analysis in 81% (17 out of 20) of urine samples of bladder tumor patients, with a specificity of 76%.

It has been demonstrated that telomerase activity is proportional to the quantity of messenger RNA of the protein component of telomerase (hTRT) (FEBS Letter (1999) 460, 285-288). Ito et al. (Clin. Can. Res. (1998) 4, 2870, 2810) have demonstrated that hTRT mRNA, the catalytic part of telomerase, is expressed in urine sediment in 76% (23 out of 33) of bladder tumor cases. The specificity of the method is 96%. Ito et al. (Clin. Can. Res. (1998) 4, 1603-1608) have provided studies on the distribution of the expression of all three components of telomerase, i.e., hTRT, hTR and TEP1, in bladder tumor tissues. hTRT is expressed in 90% of tumor tissues (30 out of 33) and in 20% of neighboring tissues. On the contrary, hTR and TEP1 are expressed both in tumor tissues and in normal tissues. Scientific literature has found no significant correlation between expression of hTRT and clinical-pathological characteristics of the tumors. Suzuki et al. (The Journal of Urology (1999) 162, 2217-2220) have demonstrated that hTRT is expressed in 100% of bladder tumor tissues (27 cases) and in none of the normal tissues that surround the tumor, while the TEP1 protein is expressed equally in tumor tissues and in normal tissues.

Disclosure of the Invention

A noninvasive method for early diagnosis of bladder tumor has now been found which is based on the simultaneous determination of the expression of the catalytic part of telomerase (hTRT) at the level of messenger RNA and of at least one additional selected molecular marker.

The diagnostic method according to the invention is based on the simultaneous determination of the messenger RNA of the protein component of telomerase, which is a marker for cell immortalization and therefore for the presence of cells in vivo which have undergone a malignant transformation, and of another selected marker.

In particular, the diagnostic method according to the invention is based on highlighting both the expression of the telomerase enzyme by quantization of the messenger RNA of the catalytic part (hTRT marker) in urine and the expression of at least one additional molecular marker, and on subsequent quantization thereof and analysis of the results obtained.

The method based on the interpretation of the response of two or more molecular markers increases sensitivity and specificity up to 100% of both. In this manner, the method according to the invention is suitable for screening and monitoring of patients undergoing treatment.

In accordance with a first aspect of the present invention, a diagnostic method for bladder tumor is provided which comprises a step of amplification of the RNA extracted from the cells present in a urine sample by using:

-- an hTRT marker;

-- a marker, preferably ß-actin, as demonstration of RNA accessibility and as standard for quantitative estimation,

in association with at least one other marker chosen from the group that comprises:

-- a marker for one of the proteins of the cytokeratin family;

-- a marker for lymphocytes which is suitable to detect inflammatory cells associated with neoplastic infiltration.

In particular, the present invention provides a method for early diagnosis of bladder tumor in a urine sample which comprises:

a) optionally, a preliminary step of extracting the total RNA from the cells that are present in the urine;

b) a step of amplification of the extracted RNA by using:

-- a marker for the messenger RNA of the catalytic component of telomerase (hTRT);

-- a marker for ß-actin

in combination with at least one additional marker chosen from the group

that consists of:

-- a marker for a protein of the cytokeratin family;

-- a lymphocyte marker which is suitable to detect inflammatory cells associated with neoplastic infiltration;

and, advantageously, a thermocycler or real-time PCR

c) a step of detecting the amplified material.

One of the markers used in the invention belongs to the cytokeratin family, which is composed of 20 polypeptides which are part of the intermediate filaments of epithelial cells. Cytokeratins are expressed in various combinations according to the type of epithelial cell and to the degree of differentiation. Among cytokeratins, the use of cytokeratin 20 (CK20) is particularly suitable in the invention.

Furthermore, it has been found that among markers for lymphocytes, CD4 is particularly suitable to detect the presence of helper lymphocytes associated with neoplastic infiltration.

In particular, determination of the RNA of hTRT in combination with three other markers, such as cytokeratin 20, CD4 and ß-actin, and subsequent coordinated interpretation of the results provide a means for a sensitive and specific diagnosis of bladder tumor directly in urine.

The step for amplification of extracted RNA according to the method of the invention advantageously comprises two steps:

i) a reverse transcription (RT) step, performed by providing a specific antisense primer or primers and

ii) an amplification step (PCR), using the respective specific sense primers, which are advantageously biotinylated in order to facilitate the subsequent detection step.

In accordance with one embodiment, the amplification step is performed with a thermocycler or real-time PCR.

According to another embodiment of the invention, the amplification step is performed in the presence of ddUTP-biotinylate.

The subsequent revelation step comprises:

-- hybridization of the products amplified in step b) in a first well which contains a probe for the amplified hTRT marker and at least one well which contains a probe for a marker for a protein of the cytokeratin family, a marker for a lymphocyte suitable to detect inflammatory cells associated with neoplastic infiltration, and ß-actin. Alternatively, the biotinylated amplified material is bound to the plate with streptavidin and the amplification products are hybridized with the probe, preferably marked with DIG.

-- detection of the bound fragment (recognition that hybridization has occurred) with the probe used, which is preferably performed by using streptavidin, which recognizes biotin and with which alkaline phosphatase or peroxidase is advantageously bound.

In accordance with another embodiment, the amplified material, hybridized with the probe marked with DIG, is detected with anti-DIG with which alkaline phophatase or peroxidase is advantageously bound. Instrumental detection, in both cases (for example with an ELISA reader), is preferably performed with the addition of a colorimetric substrate or by chemiluminescence.

As an alternative to alkaline phosphatase or peroxidase, streptavidin is marked with molecules for fluorescence emission.

In the case of use of real-time PCR, which is capable of performing amplification in real time, the detection system used can be an intercalating staining solution, such as Syber green or specific probes marked with molecules for fluorescence emission.

The diagnostic method according to the invention allows to identify neoplastic cells of epithelial origin and CD4+ lymphocytes. In particular, the combination of markers according to the invention allows to obtain detailed information regarding the desquamation of neoplastic uroepithelial cells and the infiltration of the tumor on the part of CD4+ lymphocytes. The

method according to the invention allows to analyze the cells that are present in the urine even if they are not perfectly preserved, differently from the TRAP method, which leads to a false negative if the proteins are degraded.

The method according to the invention also avoids the need to perform bladder washing, since the urine of the patient can be used directly.

This last advantage is very important, since bladder washing is in any case a diagnostic intervention performed on the patient, with additional costs and discomfort for said patient.

The method according to the invention provides a particularly accurate indication of the existence of a malignant tumor form in progress, located at the bladder, and also provides considerable advantages with respect to the other methods used so far.

According to another aspect of the invention, an apparatus for the diagnosis or for monitoring bladder tumor is provided which comprises:

-- a marker for the messenger RNA of the catalytic component of telomerase (hTRT), a marker for ß-actin to demonstrate RNA accessibility and as standard for quantitative estimation, and at least one additional marker chosen from the group that comprises:

-- a marker for a protein of the cytokeratin family;

-- a marker for a lymphocyte suitable to detect inflammatory cells associated with neoplastic infiltration.

In accordance to a preferred embodiment, said marker for a lymphocyte suitable to detect inflammatory cells associated with neoplastic infiltration is CD4, and said marker for a protein of the cytokeratin family is cytokeratin 20 or CK20.

Advantageously, the apparatus according to the invention furthermore comprises a plurality of ELISA plates and conveniently one or more of the above described reagents, markers, and probes.

The following examples are provided merely as a non-limitative

illustration of the present invention.

Example 1

A) Urine collection and storage

A human urine sample was processed according to the following procedure directly after collection:

-- collection of urine sediment by passing the urine to be tested through an appropriate filter, with application of vacuum (Talent srl);

-- the filter containing the cells was immersed in a lysis solution ("Total Quick RNA" kit by Talent), centrifuged for 1 minutes, and left immersed for 10 minutes.

The solution, after removing the filter, was stored at -80 °C.

B) Extraction of total RNA.

Extraction was performed (by means of the "Total Quick RNA" method by Talent, Trieste, Italy) by using the specific column. The RNA bound selectively with the resin, which was subsequently washed to eliminate traces of protein and DNA. The RNA was then eluted with warm water.

C) Diagnostic investigation according to the invention.

The method was conducted on an ELISA-type microplate.

I) Step of amplification of extracted RNA. This step was performed by using four specific markers and a thermocycler.

Amplification occurred in two steps:

1) reverse transcription by using a specific antisense primer

2) amplification by using the respective biotinylated specific sense primers (for the subsequent revelation procedure).

II) a detection step.

The amplification products were hybridized in four different wells. Each well contained one of the four specific probes: telomerase, CK20, CD4 and ß-actin. For each marker, positive and negative control amplification was performed, with subsequent hybridization on a microplate. The probe was complementary to the fragment amplified with the biotinylated primer.

Detection of the bound fragment occurred by means of streptavidin (which recognizes biotin), to which alkaline phosphatase was bound. After adding the colorimetric substrate (4-nitrophenyl phosphate) or chemiluminescence substrate, reading occurred with the appropriate instrument (for example with an ELISA reader).

The streptavidin was alternatively bound with a fluorochrome which was detected by virtue of an appropriate system (for example with an ELISA reader).

The cutoff levels of the CD4 and CK20 markers were chosen from the first series of experiments, using the combination of three markers (hTRT, CK20 and CD4) in a small number of cases with neoplasms and phlogosis. The cases that expressed even a minimal quantity of hTRT were considered positive. All the markers were compared with the values of ß-actin.

Interpretation of results:

The cases in which two or three markers were positive (in the case of CK20 and CD4, above the preset threshold) were considered positive (+) for tumor. Using this approach, the tumors (27 out of 31) that had already been diagnosed at the clinical level and the negative cases that also reflected the clinical diagnosis of cystitis were defined.

The cases in which only one marker of the three proposed ones (hTRT, CK20 and CD4) was positive were defined as inconclusive (10 cases). Of these, 40% (4 cases) were found to be tumors (comparing the results with clinical results) and 60% were clinically negative.

Since the tumor cases defined as inconclusive are proposed for subsequent retesting, the method according to the invention has 100% sensitivity (27 tumor cases diagnosed with our method + 4 cases defined as inconclusive). The specificity of the method was 45% (5 cases of cystitis out of 11), but if the inconclusive cases are considered, specificity is 100%. In other words, a tumor was never defined as negative and a cystitis was never considered positive.

Interpretation of the results allowed to define 3 groups:

1) Positive for at least two markers -- tumor (all cases were found to be real tumors)

2) Negative for all three markers -- absence of tumor (none of the cases defined as negative had neoplasms)

3) Inconclusive cases because only one of the markers is positive: investigation to be repeated or confirmed with other methods (of the 10 cases defined as inconclusive, 4 were then found to be tumor).

Considering the sensitivity and specificity values expressed by the individual markers, the following values were found:

1) For hTRT: 94% sensitivity, 82% specificity

2) For CK20: 74% sensitivity, 64% specificity

3) For CD4: 52% sensitivity and 100% specificity

The following Table 1 is an interpretation of the quantitative results on 42 patients (31 with tumor) expressed as sensitivity and specificity. The specificity and sensitivity of our method are compared with other noninvasive methods.

TABLE 1

Analysis	Sensitivity	Specificity
hTRT marker	94%	82%
CK20 marker	74%	64%
CD4 marker	52%	100%
Markers: hTRT + CK20 + CD4 + inconclusive cases (after second analysis performed on inconclusive cases by taking a new sample of the same patient)	100%	100%
TRAP	70%	99%
BTA	74%	73%
NMP22	53%	62%

Cytology	44%	95%
FDP	52%	95%

The following Table 2 lists the results found in 42 cases with neoplasms and phlogosis. The results show that the cases found positive simultaneously for the hTRT marker, the cytokeratin marker and the lymphocyte marker according to the present invention led to a diagnosis of bladder tumor which was confirmed clinically, indicating a high reliability of the diagnostic results found with the method according to the present invention.

TABLE 2

Cases	hTRT	CD4	CK20	Diagnosis with our method	Clinical diagnosis
1	+	-	+	Tumor	Tumor
2	-	-	-	Negative	Cystitis
3	+	+	+	Tumor	Tumor
4	-	-	+	Inconclusive	Cystitis
5	-	-	+	Inconclusive	Cystitis
6	-	-	-	Negative	Cystitis
7	+	-	-	Inconclusive	Tumor
8	+	+	-	Tumor	Tumor
9	+	-	+	Tumor	Tumor
10	-	-	-	Negative	Cystitis
11	+	-	+	Tumor	Tumor
12	+	-	+	Tumor	Tumor
13	+	-	+	Tumor	Tumor
14	+	+	-	Tumor	Tumor
15	-	-	-	Negative	Cystitis
16	-	-	+	Inconclusive	Cystitis
17	-	-	+	Inconclusive	Cystitis

18	+	-	+	Tumor	Tumor
19	+	-	-	Inconclusive	Tumor
20	-	-	+	Inconclusive	Tumor
21	+	-	+	Tumor	Tumor
22	+	-	+	Tumor	Tumor
23	+	-	-	Inconclusive	Cystitis
24	+	-	+	Tumor	Tumor
25	+	+	+	Tumor	Tumor
26	+	+	+	Tumor	Tumor
27	+	-	+	Tumor	Tumor
28	+	+	-	Tumor	Tumor
29	+	+	-	Tumor	Tumor
30	+	+	-	Tumor	Tumor
31	+	+	+	Tumor	Tumor
32	+	+	+	Tumor	Tumor
33	+	+	+	Tumor	Tumor
34	+	+	+	Tumor	Tumor
35	+	-	-	Inconclusive	Cystitis
36	+	+	-	Tumor	Tumor
37	-	+	+	Tumor	Tumor
38	-	+	+	Tumor	Tumor
39	+	-	+	Tumor	Tumor
40	+	-	+	Tumor	Tumor
41	-	-	-	Cystitis	Cystitis
42	-	+	-	Inconclusive	Tumor

Example 2

Reagents and instruments used to provide an embodiment of the method according to the invention:

<u>For RNA extraction</u>

-- absolute ethanol

-- 75% ethanol

-- microcentrifuge

-- sterile tips

For RT-PCR

-- thermocycler

For detection

-- ELISA reader

Content of an embodiment of a diagnostic kit:

1) Accessories for collecting urine sediment

2) Kit for RNA extraction

3) Kit for RT-PCR

4) Detection kit

Kit for RNA extraction

TQ-RNA Lysing solution

TQ-RNA Binding resin

TQ-RNA Washing solution A

TQ-RNA Washing solution B

TQ-RNA Conditioning solution

TQ-RNA Spin columns

2 ml collection tubes

1.5 collection tubes

Accessories for collecting urine sediment

Urine collection filters

Vacuum manifold

Kit for RT-PCR

Primer up (biotinylated)

Primer down

Reverse transcriptase

5x buffer for reverse transcriptase

10x buffer for PCR

Taq polymerase

Kit for detection

-- ELISA plates (4 plates, each containing one of the four specific probes: telomerase, CK20, CD4 and ß-actin)

-- Reagent 1 (blocking reagent)

-- Reagent 2 (hybridization solution)

-- Reagent 3A (washing solution)

-- Reagent 3B (washing solution)

-- Streptavidin, to which alkaline phosphatase is bound

-- Reagent 4

-- Reagent 5 (washing solution)

-- Reagent 6 (substrate solution)

-- Colorimetric substrate (4-nitrophenyl phosphate)

-- Reagent 7

Urine collection and storage

The urine samples must be processed as quickly as possible:

4) urine sediment was collected by vacuum and appropriate filters (supplied by Talent).

5) the filter containing the cells was immersed in the lysing solution ("Total Quick RNA" kit by Talent), centrifuged for 1 minute, and left immersed for 10 minutes.

6) the solution, after removing the filter, was stored at -80°C.

Extraction of total RNA with the "Total Quick RNA" method by Talent:

Extraction was performed with the "Total Quick RNA" method by Talent, which is a quick method (20 minutes) based on column centrifugation. The RNA was specifically bound to the resin, which was then washed to eliminate traces of protein and DNA, and then eluted with warm water.

RT-PCR

RNA concentration was measured at 260 nm absorbance. Absorbance equal to 1 unit corresponds to 40 µg of RNA per ml.

The step for amplification of the extracted RNA by using four specific markers and a thermocycler occurred in two steps:

1) reverse transcription by using a specific antisense primer (RT)

2) amplification (PCR) by using the respective specific sense primers which are biotinylated (for subsequent revelation of bound fragments).

RT

Preparation of mix:

5x buffer	2µl
Primer down (30 pmol/µl)	0.5 µl
Mixture of NTPs	1 µl
AMV	0.1 µl
RNase	0.1
H_2O	5.3 µl
RNA (500 ng/µl)	1 µl
Total volume	10 µl

Incubation at 42°C was performed for 1 hour.

PCR

Preparing the mix:

10x buffer	5 µl
Primer up (biotinylated)	0.5 µl
Taq polymerase	0.25 µl
H_2O	34.25 µl
Total volume	40 µl

The mix was added in the respective tubes of the RT and amplified by performing the following cycles:

95 °C for 3 minutes

5 cycles of:

95 °C for 1 minute

55 °C for 1 minute

72 °C for 1 minute

40 cycles of:

95 °C for 30 seconds

55 °C for 30 seconds

72 °C for 30 seconds

Detection

The amplification products were adsorbed on four different wells (for example, from a total of 50 µl, 10 µl per well were placed (each well contained one of the four specific probes: telomerase, CK20, CD4 and ß-actin). The probe was complementary to the fragment amplified with the biotinylated primer.

Detection of the bound fragment occurred by means of streptavidin (which recognizes biotin), to which the alkaline phosphatase was bound. After adding the colorimetric substrate (4-nitrophenyl phosphate), reading was performed with an ELISA reader.

The analysis procedure entailed performing the following steps:

1) 200 µl of Reagent 1 were added in the wells of the ELISA and incubation was performed at ambient temperature for one hour under agitation.

2) the amplified material was denatured at 95 °C for 10 minutes, placing it immediately in ice.

3) the wells were emptied. 90 µl of Reagent 2 and 10 µl of each amplified product were added in the respective wells, and incubation was performed at 50 °C for two hours.

4) The wells were emptied and washed 3 times with 200 µl of Reagent 3A heated at 50 °C.

5) The wells were incubated with Reagent 3A at 50 °C for 15 minutes.

6) Emptying and washing were performed 3 times with 200 µl of Reagent 3B heated to 50 °C.

7) For each well, 100 µl of streptavidin, conjugated with alkaline

phosphatase, diluted at 1:5000 in Reagent 4, were added.

8) Incubation was performed at 50 °C for 1 hour.

9) The wells were emptied and washed 3 times with 200 µl of Reagent 5 at ambient temperature.

10) The wells were incubated with Reagent 5 for 5 minutes at ambient temperature.

11) Washing was performed 3 times with 200 µl of Reagent 5 at ambient temperature.

12) 100 µl of pNPP, 10 mg/ml, dissolved in solution 6, were added.

13) Incubation was performed for 30 minutes at ambient temperature in darkness.

14) The reaction was blocked with 100 µl of Reagent 7.

15) The results were read at 405 nm.

The disclosures in Italian Patent Application No. MI2000A001002 from which this application claims priority are incorporated herein by reference.

CLAIMS

1. A method for early diagnosis of bladder tumor in a urine sample, characterized in that it comprises the determination, on the RNA extracted from the cells present in the urine, of:

-- a marker for the messenger RNA of the catalytic component of telomerase (hTRT) and a marker for ß-actin, to demonstrate RNA accessibility and as standard for quantitative estimation,

in association with at least one additional molecular marker chosen from the group that comprises:

-- a marker for a protein of the cytokeratin family;

-- a lymphocyte marker which is suitable to detect inflammatory cells associated with neoplastic infiltration.

2. A method for early diagnosis of bladder tumor in a urine sample, comprising:

a) a step of amplification of the RNA extracted from cells present in the urine by using:

-- a marker for the messenger RNA of the catalytic component of telomerase (hTRT), a marker for ß-actin to demonstrate RNA accessibility and as standard for quantitative estimation,

in combination with at least one additional marker chosen from the group that comprises:

-- a marker for a protein of the cytokeratin family;

-- a lymphocyte marker which is suitable to detect inflammatory cells associated with neoplastic infiltration;

b) a step of detecting the amplified material.

3. The method according to claim 2, characterized in that said protein marker of the cytokeratin family is cytokeratin 20 (CK20).

4. The method according to claim 2 or 3, characterized in that said lymphocyte marker suitable to detect inflammatory cells associated with the neoplastic infiltration is CD4.

5. The method according to claim 2, characterized in that said amplification comprises:

i) reverse transcription by using a specific antisense primer;

ii) amplification by using specific sense primers.

6. The method according to claim 2, characterized in that said specific sense primers of the amplification step ii) are preferably biotinylated in order to facilitate the subsequent detection step.

7. The method according to claim 2, characterized in that said detection step comprises hybridization of the amplified products with probes which are specific for the markers used in said step b) and the subsequent recognition that hybridization has occurred with said probe preferably by means of streptavidin.

8. The method according to claim 7, characterized in that said streptavidin is conjugated with alkaline phosphatase or with peroxidase for colorimetric or chemiluminescence detection.

9. The method according to claim 7, characterized in that said streptavidin is conjugated with fluorescein for fluorescence detection.

10. The method according to claim 2, characterized in that said amplification products of step b) are adsorbed on a first well which contains a probe which is immobilized for hTRT and on at least one additional well which contains a probe for the marker used in said step b).

11. The method according to any one of the preceding claims 1 to 10, characterized in that it comprises a preliminary step of extracting the total RNA from the cells that are present in the urine.

12. An apparatus for diagnosis or for monitoring bladder tumor, characterized in that it comprises:

-- a messenger RNA marker for the catalytic component of telomerase (hTRT), a ß-actin marker to demonstrate RNA accessibility and as a standard for quantitative estimation, and at least one additional marker chosen from the group that comprises:

-- a protein marker of the cytokeratin family;

-- a lymphocyte marker suitable to detect inflammatory cells associated with neoplastic infiltration.

13. The apparatus according to claim 12, characterized in that said lymphocyte marker suitable to detect inflammatory cells associated with neoplastic infiltration is CD4.

14. The apparatus according to claim 12 or 13, characterized in that said protein marker of the cytokeratin family is cytokeratin 20 or CK20.

15. The apparatus according to any one of claims 12 to 14, characterized in that it furthermore comprises a plurality of ELISA plates.

16. The apparatus according to claim 15, characterized in that it comprises 4 ELISA plates, each of which comprises one of the four specific probes: telomerase, CK20, CD4, and ß-actin.

17. The apparatus according to any one of claims 12 to 16, characterized in that it furthermore comprises streptavidin to which alkaline phosphatase or peroxidase is linked.

18. The apparatus according to any one of claims 12 to 17, characterized in that it furthermore comprises one or more washing solutions.

19. The apparatus according to any one of claims 11 to 18, characterized in that it furthermore comprises a solution for hybridization and a colorimetric substrate.

20. The apparatus according to any one of claims 12 to 16 and 18-19, characterized in that it furthermore comprises streptavidin conjugated with fluorescein for fluorescence detection.

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(54) Title: EARLY DIAGNOSIS OF BLADDER TUMOR IN URINE SAMPLES

(57) Abstract: A method for early diagnosis of bladder tumor in a urine sample, which comprises a step of amplification of the RNA extracted from cells present in the urine by using a marker for the messenger RNA of the catalytic component of telomerase (hTRT), a marker for β-actin to demonstrate RNA accessibility and as standard for quantitative estimation, in combination with at least one additional marker chosen from the group that comprises: a marker for a protein of the cytokeratin family, a lymphocyte marker which is suitable to detect inflammatory cells associated with neoplastic infiltration, and a final step of detecting the amplified material.

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B. FIELDS SEARCHED

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Electronic data base consulted during the international search (name of data base and, where practical, search terms used)
EPO-Internal, BIOSIS, CHEM ABS Data, MEDLINE, WPI Data, PAJ

C. DOCUMENTS CONSIDERED TO BE RELEVANT

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[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

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C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

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